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SHORT COMMUNICATION

Phylogenetic Relationship and Inter-island Genetic Variation in *Malaxis* (Orchidaceae), Endemic to the Bonin Islands

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We examined the inter-island genetic variation and phylogenetic relationship in *Malaxis boninensis* and *M. hahajimensis*, endemic to the Bonin (Ogasawara) Islands, based on sequences of the *matK*, the *trnL* intron and *trnL-trnF* spacer region and the ITS region. The phylogenetic tree indicated that the two Bonin species are closely related to each other. In the two species, two haplotypes (northern and southern types) were recognized from the sequences of chloroplast DNA, but the ITS sequences were identical. The northern type was found in plants of *M. boninensis* from Chichi-jima; the southern type was shared between plants of *M. boninensis* from Kita-Iwo-jima and *M. hahajimensis*, endemic to Haha-jima. Genetic as well as morphological variation should be considered in conservation measures to protect *Malaxis* in the Bonin Islands.

Key words: Bonin Islands, inter-island genetic variation, *Malaxis*, Ogasawara, Orchidaceae, phylogeny

The genus *Malaxis* Sol. ex. Sw., consisting over 300 species, is one of three major genera, including *Liparis* Rich. and *Oberonia* Lindl., in the tribe Malaxideae (Epidendroideae, Orchidaceae). The discrimination between *Malaxis* and *Liparis* is often unclear, but *Malaxis* is mostly distinguished from other genera by floral morphology. The ovary in *Malaxis* is twisted 360° and the flowers are non-resupinate with a short column. The tribe Malaxideae has a cosmopolitan distribution, and *Malaxis* is nearly cosmopolitan, distributed mainly from southeastern and southern Asia to Micronesia, Melanesia and Polynesia (Rasmussen 1985). Recent molecular phylogenetic study of Malaxideae using *matK* and ITS sequences indicated that *Malaxis* is not monophyletic because two terrestrial lineages of *Liparis* are nested within it, and that *Malaxis* is divided into two lineages with *Liparis*, which is congruent with two types of leaves, plicate and conduplicate (Cameron 2005).

The Bonin (Ogasawara) Islands are of recent volcanic origin in the western Pacific Ocean and consists of four island groups from north to south: Muko-jima-gunto, Chichi-jima-gunto, Haha-jima-gunto, and Iwo-jima-gunto (Fig. 1). In the Bonin Islands, there are many endemic and endangered species (Toyoda 2003, Yahara 2003). Except in the northernmost Muko-jima-gunto, two evergreen, perennial, terrestrial, endemic species of *Malaxis*, *M. boninensis* (Koidz.) K. Nakaj. and *M. hahajimensis* S. Kobay. are distributed. These two species are distinguished from each other based on leaf, inflorescence and floral morphologies (Kobayashi 1980, Ono & Kobayashi 1985, Toyoda 2003): *Malaxis boninensis* has lanceolate-oblong to elliptic leaves, a dense raceme with 25 to 30 pale green flowers, and a pale green trilobed lip with an acute small tooth on each side of the base of the apical lobe. *Malaxis hahajimensis* has ovate to broadly elliptic leaves, a loose raceme with 15 to 25 pale green flowers bearing a purplish lip without teeth between the lobes. Different chromosome numbers for the two species have also been reported, $2n = 38$ for a plant of *M. boninensis* from Chichi-jima and $2n = 36$ for a plant of *M. hahajimensis* from Haha-jima (Ono & Masuda 1981, Ono & Kobayashi 1985). Based on their similar morphologies, it was thought that these two species are closely related to each other (Ono & Kobayashi 1985). The Bonin species show an allopatric distribution in the Islands. *Malaxis boninensis* occurs in a limited, moist area on Chichi-jima and is often considered to be endemic to the Island; *M. hahajimensis* is endemic to Haha-jima (Kobayashi 1994). *Malaxis boninensis* was also reported from Iwo-jima and Kita-Iwo-jima (Ono & Kobayashi 1985, Kobayashi 1988, Fujita *et al.* 2004). In the herbarium of the University of Tokyo (TI), we found a specimen from Kita-Iwo-jima collected by Prof. T. Tuyama in 1935.

Kobayashi (1988) believed that the Bonin species to be related to the *Malaxis biloba* (Lindl.) Seidenf. & Smitin. (= *M. acuminata* D. Don) group of the Himalaya, China, Sumatra and Java, with the Bonin Islands being the northeastern end of the range of the group. She suspected that *Malaxis* in the Bonin Islands had a recent common ancestor, and that they separately differentiated under different environments on Chichi-jima and Haha-jima (Kobayashi 1988). Kobayashi (1994) later reported that *M. boninensis* was closely related to *M. alamaganensis* S. Kobay. from the Northern Mariana Islands in Micronesia instead of to the Asian species. The hypothesis, however, was based on morpho-

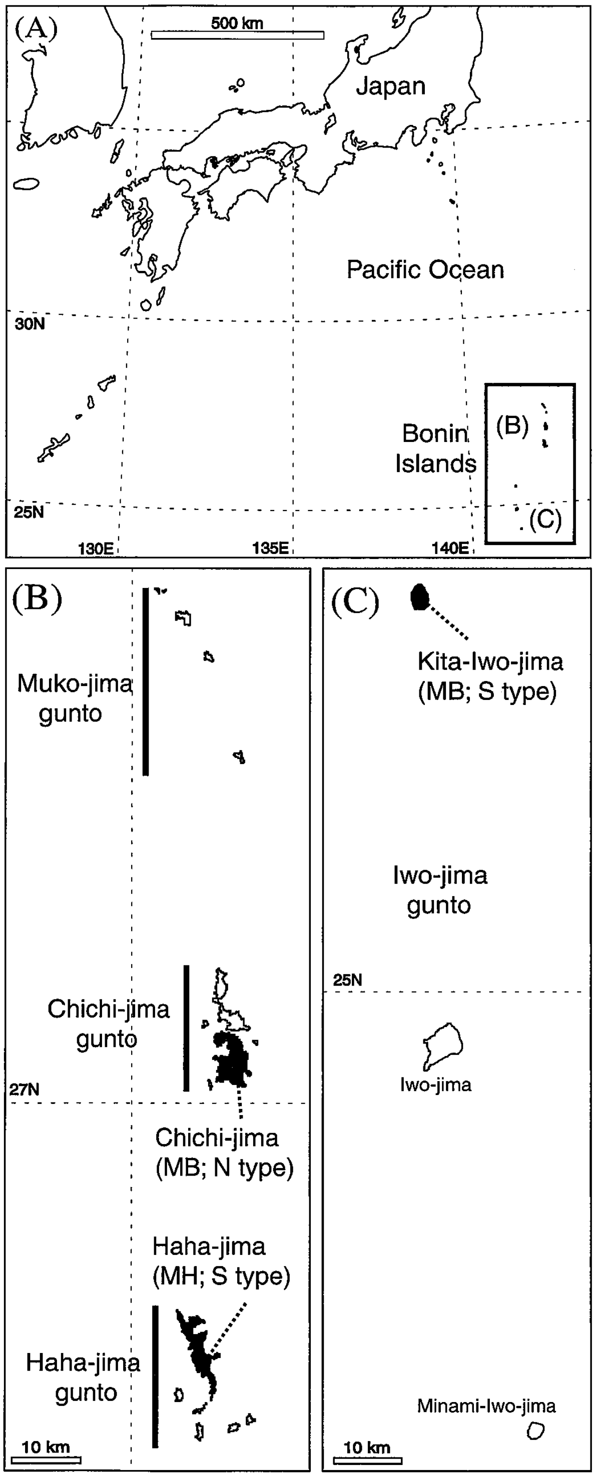

FIG. 1. Geographical distribution of the species of *Malaxis* and chloroplast DNA types. (A) Location of the Bonin Islands; (B) Northern three island groups; (C) Iwo-jima-gunto *Malaxis* in the Bonin Islands occurs on shaded islands. Abbreviations of species (MB = *M. boninensis*, MH = *M. hahajimensis*) and chloroplast DNA types (N type = northern type, S type = southern type) are shown in parentheses with the island name.

logical evidence, and the phylogenetic relationship of these species was unknown.

In this study, we examined the phylogenetic relationship and inter-island genetic variation in *Malaxis boninensis* and *M. hahajimensis* based on DNA sequences. We used two plants each of *M. boninensis* from Chichi-jima and *M. hahajimensis* from Haha-jima, which were cultivated in the Botanical Gardens of the University of Tokyo (Appendix 1). The species of *Malaxis* in the Bonin Islands are now in danger of extinction because of their small numbers and they are assigned to category CR in the Red Data Book (Yahara 2003). These two species, as well as several other endangered species endemic to the Bonin Islands, have been conserved in the Botanical Gardens of the University of Tokyo. Recently, we also obtained three plants of *Malaxis* from Kita-Iwo-jima and identified them as *M. boninensis*, based on the above-mentioned morphological characters, and have placed them in the Botanical Gardens of the University of Tokyo and in the experimental greenhouse of Tokyo Metropolitan University for conservation (Appendix 1).

For the molecular analysis, we included other plants of *Malaxis*, including *M. acuminata*, which Kobayashi (1988) considered to be closely related to the Bonin species (Appendix 1). From a fresh leaf of each plant, total DNA wais extracted by the CTAB method (Doyle & Doyle 1987) with slight modifications. The sequences of the *matK* gene (Cameron 2005, Topik *et al.* 2005) and the *trnL* intron and *trnL-trnF* spacer region (*trnLLF*) region (Taberlet *et al.* 1991) in the chloroplast DNA (cpDNA) and the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (White *et al.* 1990, Topik *et al.* 2005) were determined by PCR amplification and direct sequencing. The sequences have been registered in the DNA Data Bank of Japan (DDBJ) and linked with GenBank; their accession numbers are shown in Appendix 1. In multiple alignments on using the software GENETYX-MAC, we compared sequences among seven plants of the Bonin species. Although no mutations were observed in the ITS sequences, one substitution was found in each of the *matK* (1412 bp) and *trnLLF* (1224 bp) sequences. As a result, two haplotypes were distinguished based on the differences in the two substitutions in the combined sequences of cpDNA. One type (the northern type) was found in plants of *M. boninensis* from Chichi-jima; another type (the southern type) was shared among plants of *M. hahajimensis* from Haha-jima and *M. boninensis* from Kita-Iwo-jima (Fig. 1). No clear morphological differences between plants of *M. boninensis* from the north and south were found.

The phylogenetic analyses were conducted by maximum parsimony analysis using PAUP (Swofford 2002). Although the *matK* and ITS sequences of many species in Malaxideae are registered in GenBank by Cameron (2005), the sequence of *trnLLF* from GenBank is available only for a single species. Therefore, we conducted analyses based on sequences of *matK* and ITS. Preliminarily, to determine which lineages of the tribe Malaxideae detected in Cameron (2005) the species of *Malaxis* from the Bonin Islands belong, we analyzed a large data set of of *matK* and ITS sequences. As a result, both the *matK* and ITS trees indicated that the species from the Bonin Islands are included in the lineage of *Malaxis* with conduplicate leaves, which was determined by Cameron (2005) (data not shown). We then analyzed the relationships within the lineage of *Malaxis* with conduplicate leaves using combined sequences of *matK* and ITS. The phylogenetic tree obtained indicated that two species of *Malaxis* from the Bonin Islands are closest to each other (Fig. 2). *Malaxis acuminata* from Nepal, which was considered to be close to the Bonin species by Kobayashi (1988), is not related to them. The clade including the Bonin species may be sister to the clade of *M. oculata* Kuntze and *M. punctata* J. J. Wood from Malaysia in Southeast Asia, but the reliability is low (bootstrap probability 53%). Because the branch length of the Bonin clade is relatively long (12 steps), their species closest to them are not clear. Studies including more species of *Malaxis*, especially from Micronesia, are needed to determine the origin of the Bonin species.

Sister relationship and inter-island genetic variation were detected in *Malaxis* in the Bonin Islands based on molecular data. Additionally, we also noted that plants from Haha-jima (*M. hahajimensis*) and those from Kita-Iwo-jima (*M. boninensis*) shared the same cpDNA haplotype. The geographical patterns in inter-island genetic and morphological variations in the Bonin species are incongruent. Although we have insufficient evidence to discuss the evolution of this situation, isolation of the populations on the three islands and morphological diversification between the Haha-jima population and the others and genetic diversification of the cpDNA between the Chichi-jima population and the others might have occurred independently after migration to the Bonin Islands of their common ancestor. In contrast, the geographical patterns in genetic and morphological variations are congruent in another endemic species, *Hibiscus glaber* Matsum. ex Nakai (Takayama *et al.* 2005). Thus, geographical patterns in genetic and morphological variations vary by species and reflect different evolutional histories. In considering the conservation of endemic and endangered plants in the Bonin Islands we should consider local genetic as well as morphological variation.

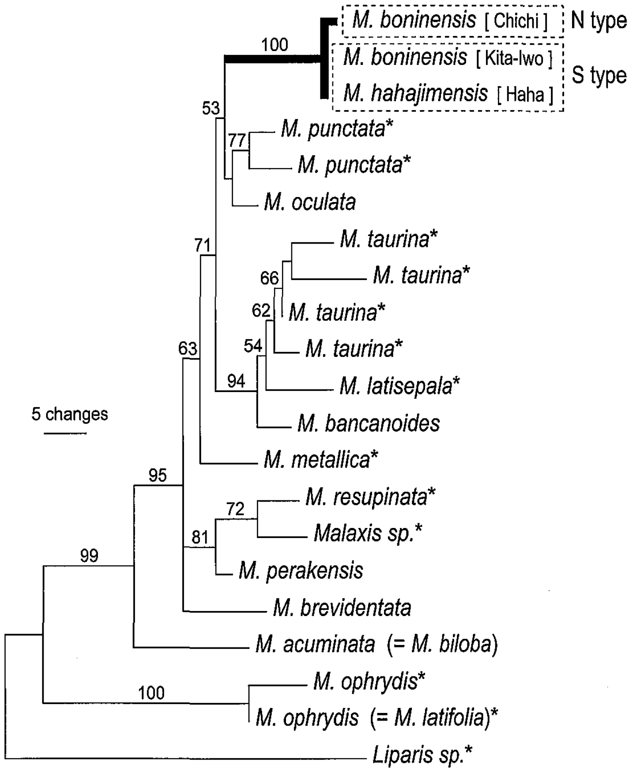

FIG. 2. Phylogenetic tree of the lineage of *Malaxis* with conduplicate leaves. Strict consensus tree was constructed from three most parsimonious trees (tree length = 189, consistency index = 0.9153, rescaled consistency index = 0.8238) obtained based on combined sequences of *matK* and ITS. Sequences of species with asterisks are from GenBank; accession numbers refer to Cameron (2005). *Liparis sp.* was used as the outgroup species in this tree. Search for most parsimonious tree was conducted by branch and bound search on parsimony analysis. DELTRAN character optimization option was used to construct the strict consensus tree. Values on branches are bootstrap probabilities, which were examined by 1,000 permutations with the same tree search procedure option.

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APPENDIX 1. List of samples analyzed in this study: taxon, sample and voucher number, source, and DDBJ accession numbers in parentheses.

Malaxis acuminata D. Don.: *M. Watanabe s. n., TBG 84561* (TNS) from Nagurjum, Nepal (*matK* AB290892, ITS AB290884).

M. bancanoides Ames: *Yukawa 95-101* (TNS), Iriomote-jima, Japan (*matK* AB290893, ITS AB290885).

M. boninensis (Koidz.) K. Nakaj.: *Ohi-Toma C01* (TI), *BGUT 04-189*, and *Ohi-Toma C03* (TI), *BGUT 99-283*, from Chichi-jima (*matK* AB290895, *trnLLF* AB290900, ITS AB290887); *Ohi-Toma Iw01* (TI), *BGUT 200704*, *Ohi-Toma Iw02* (TI), *BGUT 200705*, and *Kato 060006* (MAK), from Kita-Iwo-jima (*matK* AB290897, *trnLLF* AB290901, ITS AB290889).

M. brevidentata C. Schweinf.: *Yukawa 97-2066* (TNS), Espiritu Santo, Vanuatu (*matK* AB290894, ITS AB290886).

M. hahajimensis S. Kobay.: *Ohi-Toma H01* (TI), *BGUT 00-358*, and *Ohi-Toma H02* (TI), *BGUT 01-125*, from Haha-jima (*matK* AB290896, *trnLLF* AB290902, ITS AB290888).

M. oculata Kuntze: *Nazarudin s. n.* (TNS), Malay Peninsula (*matK* AB290898, ITS AB290890).

M. perakensis (Ridl.) Holttum: *Nazarudin s. n.* (TNS), Malay Peninsula (*matK* AB290899, ITS AB290891).